Sex attraction in *Polistes dominulus* (Christ) demonstrated using olfactometers and morphological source extracts

JOHN K. MACKENZIE[1], PETER J. LANDOLT[2,4] and RICHARD S. ZACK[3]

ABSTRACT

Y-tube and parallel tube olfactometers were used to test for attraction between and within genders of the European paper wasp, *Polistes dominulus* (Christ). In the Y-tube olfactometer, unmated females were attracted to male odour, while males were repelled by unmated female odour. Males and females were not attracted to the odour of the same sex in this experiment. In the parallel tube olfactometer, females were attracted to male odour, while males were not attracted to female odour. Morphological sources of potential sex attractants were tested using an arena bioassay design. Males rubbed their mandibles and gaster on the substrate when exposed to extracts of unmated female or male tagmata, female or male legs, or the male seventh gastral sternite. We did not see overt behavioural responses by females to male or female extracts.

Key Words: attractant, pheromone, *Polistes dominulus*, paper wasp

INTRODUCTION

Sex pheromones are chemicals that elicit behaviour related to mate-finding, mate-selection, and copulation in insects, including vespid wasps (Wilson 1971; Shorey 1977; Landolt et al. 1998). Close-range attractants and copulatory incitants or aphrodisiacs have been demonstrated between males and females of the social wasps *Polistes exclamans* Viereck (Post and Jeanne 1984; Reed and Landolt 1990a), *Polistes fuscatus* (F.) (Post and Jeanne 1983a, 1984), *Belonogaster petiolata* Degeer (Keeping et al. 1986), *Vespula squamosa* Drury (Reed and Landolt 1990b), and *Vespa* spp. (Batra 1980; Ono and Sasaki 1987). Despite such demonstrations, no vespid sex pheromone chemical structure has been identified. Sexual behaviour of *Polistes dominulus* (Christ) (Hymenoptera: Vespidae) has not been described or quantified in controlled experiments. Knowledge of behavioural responses to putative pheromones is necessary for accurate pheromone characterization.

Behavioural evidence in *Polistes* paper wasps suggests that sex pheromones from exocrine glands in the mandibles, legs, and gastral sterna may be involved in mate attraction (Landolt and Akre 1979; Jeanne *et al.* 1983; Beani and Turillazzi 1988; Beani and Calloni 1991a,b; Beani *et al.* 1992). In several of these species, mating often occurs away from the nest (Noonan 1978) on perching substrates that are at prominent locations such as on hilltops (Beani and Turillazzi 1988; Mathes-Sears and Alcock 1986). Males, in some species of *Polistes*, scent-mark by dragging their posterior gastral sternites (Post and Jeanne 1983b; Reed and Landolt 1991) and by rubbing their mandibles (Wenzel 1987; Reed and Landolt 1991) on the perching substrate. Four spe-

[1] 4316 Pioneer Court, Abbotsville, BC, Canada V2S 7Z1
[2] USDA-ARS, 5230 Konnowac Pass Road, Wapato WA 98951, USA
[3] Department of Entomology, Washington State University, Pullman WA 99164, USA
[4] Correspondence: USDA, ARS, 5230 Konnowac Pass Road, Wapato, WA 98951 USA, Tel. 509 454 6570, fax 509 454 5646, e-mail peter.landolt@ars.usda.gov

cies, including *P. dominulus*, are shown to drag their hind legs on the substrate (Beani and Calloni 1991a). *Polistes dominulus* males have ducted class III gland cells that open onto the cuticle in their legs, as well as onto the seventh abdominal sternite (Downing *et al.* 1985; Beani and Calloni 1991a). Although females of *Polistes* spp. are not known to show overt scent-marking behaviour, *P. exclamans* females possess a pheromone in the venom that elicits sexual behaviour in both conspecific and heterospecific males (Post and Jeanne 1984). Additionally, a surface pheromone on the thoracic and gastral cuticle in *P. fuscatus* is important for male recognition of conspecific females (Post and Jeanne 1984).

The objective of this study was to investigate orientation and behavioural responses of *P. dominulus* to potential sex odours from a variety of morphological sources. Klinotaxic (turning orientation) and orthotaxic (forward orientation) (Fraenkel and Gunn 1940; Wyatt 2003) responses to male odour and unmated female odour were tested using Y-tube and parallel tube olfactometers, respectively. An arena-type bioassay was used to test for behavioural responses to extracts of male and unmated female tagmata and glands. These studies are foundational in aiding the overarching objectives of determining sex pheromone signaling systems in this species, and determining sources of those sex pheromones.

MATERIALS AND METHODS

Colony Collection and Maintenance. *Polistes dominulus* nests with pupae were collected in the field, and placed in plastic screened cages (30.5 x 30.5 x 30.5 cm) during late August and early September in 2003 and 2004. Nests were collected at this time because males were abundant, indicating that reproductive females would be emerging from nests, and not worker females. Collected nests were monitored daily to remove and segregate male and female adults that emerged, in order to minimize encounters between the sexes and exposure to sex pheromones. Male and female wasps obtained in this manner were assumed to be sexually inexperienced since mating is reported to occur away from the nest (Noonan 1978). These wasps were used for the preparation of extracts and for behavioral assays conducted in Pullman and Yakima, Washington.

In Pullman (Whitman County), Washington, USA, the newly emerged wasps were kept in a laboratory at 24°C, 40% RH, under a natural light regime (14 hours of light and 10 hours of dark), until testing in the Y-tube olfactometer and in the parallel tube olfactometer. All Y-tube olfactometer tests were conducted in Pullman, while one half of bioassay replicates for each experiment conducted with parallel tube olfactometers were conducted in Pullman and the other half at the USDA, ARS Yakima Agricultural Research Laboratory near Yakima (Yakima County), Washington, USA. Wasps used in olfactometer tests and the arena bioassays conducted in Yakima were kept in a glass greenhouse under natural lighting at 30 ± 3 °C and 35% RH. At both sites, wasps in cages were provided water and a 1:10 molasses:water solution on cotton balls for nutrition. Water and the solution of molasses were refreshed or replaced daily. Wasps used in all assays were between 2 and 14 days old; they were randomly selected for each trial and were not reused in other trials for at least 48 hours.

Y-tube Olfactometer Bioassay. Unmated male and female wasps were tested for klinotaxic responses to unmated female odour and male odour in the Y-tube olfactometer. The inside diameter of the glass Y-tube was 2.5 cm and the length of the tube, from stem base to Y-juncture, was 18 cm. Airflow of 100 ml/min was measured with a flowmeter (Aalborg Instruments, Monsey, NY) before and after passing through the 480 ml jars housing the treatments. Air passed through the treatment jar containing 3 "bait wasps" of the sex being assayed, then through one arm of the Y-tube, and out the stem of the Y-tube. Simultaneously, air

passed through the empty control jar, through the other arm of the Y-tube, and out the stem of the Y-tube.

A paper wasp was placed in the stem of the Y-tube and observed for a maximum of five minutes. If the wasp moved upwind to the Y-juncture and then moved completely beyond the juncture into either of the arms (with treatment airflow or with control airflow), that assay was ended and the response was recorded. Ten wasps were tested individually and in succession using the same "bait wasps" in the treatment jar. To eliminate a potential left or right turning bias, the positioning of the treatment and control was switched after the first 5 wasps had been tested. A clean olfactometer system was then set up and a fresh set of bait wasps was placed in the treatment jar. This experimental protocol was conducted four times to provide a total of 40 wasps (5 wasps in series x 2 treatment positions x 4 = 40) tested for responses. Wasps entering the treatment arm, the control arm, or neither arm of the Y-tube were recorded. For each experiment, the numbers of wasps that entered the treatment arm or the control arm were compared using the Chi-square goodness-of-fit test with Yates correction for continuity at $P \leq 0.05$ (Zar, 1974).

The olfactometer system was placed horizontally 50 cm beneath two 1.2 m long, 34W fluorescent bulbs (Osram Sylvania Corp., Danvers, MA) and one 160W mercury vapour bulb (Osram Sylvania Corp., Danvers, MA). Temperature at the olfactometer surface was 31°C. Air moving through the Y-tube olfactometer was supplied by an aquarium air pump, purified through a hydrocarbon trap (Alltech Associates Inc., Deerfield, IL), and humidified with a gas diffusion bottle. All glassware (Ace Glass, Inc. Vineland, NJ) and steel tubing were washed in hot water with Micro-90 cleaning solution (International Products Corp., Burlington, NJ), and then rinsed serially with deionized water, acetone, and then hexane. Glassware was subsequently placed in a drying oven at 180°C overnight before used again in assays.

Parallel Tube Olfactometer Bioassay. Unmated females were tested for orthotaxic responses to male odour and males were tested for orthotaxic responses to unmated female odour in a parallel or "straight tube" olfactometer design. This design is based on that of Tobin *et al* (1981) and was reported by Landolt *et al.* (1988). The olfactometer set up was the same as the Y-tube set up, except for the replacement of the Y-tube with two straight glass tubes. Each straight tube was supplied 100 ml/min of metered, purified humidified airflow that was passed through a glass jar housing an odour source, separate from the other tube and odour source. This setup was placed on a laboratory table with fluorescent lighting above and natural lighting from windows. A wasp was placed in a straight glass tube, 2.5 cm diameter and 18 cm long, downwind from the treatment airflow (3 bait wasps) and another wasp was placed in an identical tube downwind from the control airflow (empty). For each wasp, the time it took to cover the full 18 cm distance of the tube was recorded, if indeed it completed the full distance upwind. This assay was conducted with 10 pairs of wasps (treatment and controls paired), and the glassware for treatment and control were switched after 5 pairs of wasps were tested. This experiment was then replicated eight times, (N = 80) and treatment mean times were separated from control mean times using a paired *t*-test at $P \leq 0.05$. Also, the mean percents of those that travelled the entire lengths of the treatment and control tubes, within the five minute time limit, were separated using a paired *t*-test at $P \leq 0.05$.

One half of the parallel tube olfactometer replicates were conducted under the same conditions as the Y-tube olfactometer bioassays, in Pullman. The other replicates, in Yakima, were conducted as they were in Pullman, with these slight modifications: (1) the bioassays were conducted in a controlled environment room at 24 °C and 65% RH; (2) airflow was from a compressed air source; (3) light was supplied by two, 1.2 m long, 34 W fluorescent light bulbs (Osram Sylvania Corp., Danvers, MA) 50 cm above the olfactometer. A J16 Digital Photometer

(Tektronix Inc., Beaverton, OR) measured at 27,663 lux (lumens/m^2) at the olfactometer surface; (4) after cleaning, the olfactometer glassware and tubing were placed in a drying oven for 24 h. Data from the parallel tube olfactometer bioassays in Pullman and Yakima were pooled and analyzed together. We did not expect the minor differences in assay conditions to alter the behaviour of the wasps and a preliminary analysis of the results indicated similar responses in the assays.

Tagmata and Extract Preparations. Dissecting and grinding tools and equipment were washed in hot water with Micro-90 cleaning solution, and then rinsed with deionized water, acetone, and methylene chloride. Samples of 40 female heads, 40 male heads, 40 female thoraces, 40 male thoraces, 40 female gasters, and 40 male gasters, all from freshly freeze-killed wasps, were each ground with a mortar and pestle in methylene chloride. Additionally, 40 female venom sacs with acid sting glands, 40 female alkaline glands, legs of 40 females, 40 male mandibles with ectal glands, 40 male seventh gastral sternites with glands, and legs of 40 males were dissected or removed and then extracted with methylene chloride. All tagmata and gland extracts were reduced to 4 ml under a N_2 stream and kept in a freezer at -15 °C, providing concentrations of one wasp-equivalent per 100 µL of extract.

Tagmata and Gland Bioassay. Arena bioassays were conducted in the same greenhouse environment in which the wasps were housed. The assays occurred over the course of 3 weeks in September between 10:00 and 16:00 hr. Light intensity at the table was 16,758 ± 795 (mean ± S.E.) lux, measured at 20 different times throughout the bioassays.

On a table covered with white paper, a wasp was placed under the bottom half of an upside down, plastic, 8.5 cm diam. Petri dish for one minute before experiencing extract odour. Petri dishes and paper were discarded after each bioassay. Immediately prior to conducting the assay, 100 µl of the treatment extract or 100 µl of the methylene chloride control were applied to ¼ wedges of 5.5 cm diameter, #3 Whatman Filter Paper (Whatman International Ltd., Maidstone, England). The methylene chloride was evaporated before the filter paper was placed under the Petri dish with the wasp. Wasps were observed for two minutes while in the presence of the extract or solvent blank, after which they were placed into holding cages to ensure they were not used again in the assay. At the end of the assay period on any given day, all wasps were returned to cages that constituted the general pools of male and female wasps from which random selections were made for subsequent experiments.

Male and female wasps were tested for responses to extracts of tagmata and glands from both sexes. Each of the tests was replicated 20 times. In bioassays of male and female tagmata, the sequence was: blank, head, thorax, and gaster. In bioassays of female gland bioassay the sequence was: blank, venom, legs, and alkaline gland. In bioassays of male glands the sequence was: blank, mandibles, legs, and seventh sternal gland. For each assay, a record was kept of continuous movement, no movement, and the number of times a wasp showed stop & go movement, antennal contact with the filter paper, grooming fore legs through mandibles and then rubbing antennae, grooming fore legs through mandibles then rubbing thorax, grooming gaster with hind legs, rubbing hind legs together, rubbing mandibles on substrate, and rubbing gaster on substrate. For each behaviour and each sex, a 2x2 contingency table was constructed to make comparisons of the number of times a behaviour was observed for the control versus each of the tagmata and gland extracts. For each behavior, contingency tables were analyzed using the Chi-square statistic with Yates correction for continuity at $P \leq 0.05$ (Zar, 1974).

RESULTS

Y-tube Olfactometer Bioassays (Table 1). Females significantly more often turned toward male odour and away from the control ($P < 0.001$). Males significantly more often turned away from female odour and toward the control ($P < 0.05$). Neither males nor unmated females turned toward odour from males ($P > 0.10$) and females ($P > 0.5$), respectively, compared to the control.

Parallel Tube Olfactometer Bioassays. The mean time (± S.E.) of female movement toward male odour (33.7 ± 4.4 sec, df = 79, $P = 0.002$) was significantly lower than toward the control (49.1 ± 4.1 sec). There was no significant difference in the mean percent (± S.E.) of females that traveled the length of the treatment (89.0 ± 4.3%, df = 7, $P = 0.087$) and control (81.0 ± 4.3%) tubes within five minutes. The mean time (± S.E.) for male movement toward female odour (88.2 ± 14.3 sec, df = 79, $P = 0.141$) was not statistically different compared to the control (68.1 ± 12.0 sec). This was also true for the mean percent (± S.E.) of males that travelled the length of the treatment (70.0 ± 7.3%, df = 7, $P = 0.361$) and control (78.8 ± 6.9%) tubes within five minutes.

Tagmata Experiments. Females did not behave differently in the presence of extracts of female or male tagmata compared to the blank. Males rubbed their mandibles on the substrate more often when treated with extract of ♀ thorax ($P < 0.05$) compared to the blank (Table 2). They also rubbed their gaster on the substrate more often when treated with extracts of ♀ thorax ($P < 0.005$) or ♀ gaster ($P < 0.05$) compared to the blank (Table 2). When presented with extracts of ♂ thorax and ♂ gaster, males rubbed their mandibles on the substrate more often ($P < 0.05$) compared to the blank (Table 2).

Gland Experiments. Unmated females did not show any significant behavioural differences in the presence of extracts of female or male glands compared to the blank. Males rubbed their mandibles ($P < 0.05$) and gaster ($P < 0.005$) on the substrate more often when exposed to ♀ leg extract (Table 3). When treated with ♂ leg extract, males continuously moved ($P < 0.001$) and rubbed their mandibles on the substrate ($P < 0.05$). They also rubbed their mandibles ($P < 0.005$) and gaster ($P < 0.005$) on the substrate when exposed to ♂ seventh sternite with gland extract (Table 3). Lastly, males rubbed their gaster on the substrate ($P < 0.005$) more often when exposed to ♂ mandibles with ectal mandibular gland extract (Table 3).

DISCUSSION

Polistes males are known to use mating strategies such as marking behaviour by rubbing their mandibles, gaster, and legs on perch sites (Beani and Calloni 1991a; Polak 1993). It is hypothesized that such scent-marked perch sites attract females (Landolt and Akre 1979; Post and Jeanne 1983b,c; Wenzel 1987; Reed and Landolt 1990a). Our orientation bioassay results using olfactometers support the hypothesis that females are attracted to a pheromone of males since male odour elicited positive turning and increased speed of forward movement from females. Female attraction to males in olfactometers has also been shown for the paper wasp species *P. fuscatus* (Post and Jeanne 1983a) and *P. exclamans* (Reed and Landolt 1990a).

The observed repellency of females to males in the Y-tube, but not the parallel-tube olfactometer, assays is puzzling and calls for possible explanation. It is assumed that females would control release of any sex attractant or courtship pheromone and we have no means of knowing if and when females were "calling" when they were tested in olfactometers. Thus, females may be attractive to males under circumstances not met by our assay conditions. Also, female venom may possess alarm pheromone (Bruschini et al. 2006), which could complicate assay results when females have

Table 1.

Numbers of European paper wasps, *Polistes dominulus*, turning towards airflow from over conspecific wasps compared to control airflow, in a Y-tube olfactometer[1].

Bioassay	$N_{treatment}$	$N_{control}$	$\chi^2_{experimental}$	*P*-value
♀ to ♀	22	18	0.225	$P > 0.5$
♀ to ♂	31	9	11.025	$P < 0.001$
♂ to ♀	13	27	4.225	$P < 0.05$
♂ to ♂	16	24	1.225	$P > 0.10$

[1] Analyzed using Chi-square goodness-of-fit test with Yates correction for continuity where $\chi^2_{theoretical(1, 0.05)} = 3.841$.

Table 2.

Numbers of unmated male European paper wasps, *Polistes dominulus*, responding to unmated male and female tagmata in an arena type assay[1].

Behaviour[2]	Blank	♀Head	♀Thorax	♀Gaster	♂Head	♂Thorax	♂Gaster
A	12	14	15	17	11	11	14
B	3	4	0	2	4	2	2
C	5	2	5	1	5	7	4
D	5	8	10	10	4	4	10
E	12	8	13	8	7	10	7
F	5	0	7	3	4	6	8
G	2	4	4	3	3	3	3
H	4	0	5	1	0	4	4
I	2	7	9*	7	4	9*	9*
J	0	2	9	6*	2	3	4

[1] Within a row, treatments compared only to blank in a 2x2 contingency table analyzed using the Chi-square statistic with Yates correction for continuity are significant at $P \leq 0.05$. Numbers with an asterisk are significantly different from the blank.

[2] A – continuous movement, B – no movement, C – stop & go, D – antennate paper, E – groom fore legs in mandibles; rub antenna, F – groom fore legs in mandibles; rub thorax, G – groom gaster with hind legs, H – rub hind legs together, I – rub mandibles on substrate, J – rub gaster on substrate.

been handled and disturbed. Caution must then be exercised in interpreting negative or apparently conflicting results.

Female *P. dominulus* in this study showed neither a positive nor negative orientation response toward female odour. Overwintering females in search of hibernation sites might be expected to respond to aggregations of females (Reed and Landolt 1991). Heterospecific aggregations of paper wasp gynes in hibernacula have been reported (Rau 1930, 1938; Bohart 1942; Hermann *et al.* 1974; Gibo 1980; Reed and Landolt 1991) as well as purely conspecific aggregations (Rau 1938; Strassmann 1979). *Polistes dominulus* queens appear to overwinter in multi-colony groups (Starks 2003). However, there may be multiple factors (such as temperature and day length) that stimulate females to seek out hibernacula and other overwintering females. The wasps used in these bioassays were housed under summer-like temperatures and day length. Hence, we can make no conclusions regarding the presence or absence of a female aggregation pheromone.

Venom is thought to include a sex pheromone that elicits copulatory behaviour in males of *P. fuscatus* and *P. exclamans*

Table 3.

Numbers of unmated male European paper wasps, *Polistes dominulus*, responding to unmated male and female glands in an arena assay[1].

Behaviour[2]	Blank	♀Venom	♀Legs	♂Mandibles	♂Legs	♂Sternite	Blank	♀Alkaline
A	8	13	14	15	19**	15	11	12
B	3	1	2	1	0	2	2	5
C	9	6	4	4	1	3	7	3
D	6	11	11	13	12	13	5	7
E	9	8	15	14	9	9	11	12
F	1	0	3	2	2	1	4	2
G	6	2	10	7	6	6	7	5
H	4	2	7	6	4	5	4	6
I	2	6	9*	6	9*	12*	4	3
J	1	4	10*	9*	5	10*	3	5

[1] Within a row, treatments compared only to blank in a 2x2 contingency table analyzed using the Chi-square statistic with Yates correction for continuity (significant at $P \leq 0.05$, $df = 1$). Numbers of responses with an asterisk are significantly different from the blank.

[2] A – continuous movement, B – no movement, C – stop & go, D – antennate paper, E – groom fore legs in mandibles; rub antenna, – groom fore legs in mandibles; rub thorax, G – groom gaster with hind legs, H – rub hind legs together, I – rub mandibles on substrate, J – rub gaster on substrate

(Post and Jeanne 1983a; 1984). Odorants on the female cuticle are thought also to be a conspecific sex pheromone in these species (Post and Jeanne 1983a, 1984). Our olfactometer bioassays did not evaluate copulatory behaviour, but rather orientation behaviour. Males of *P. dominulus* did not orient by turning or increasing their speed of movement toward female odour in either the Y-tube or parallel tube bioassays, respectively. We did observe behavioural responses such as increased movement and possible scent marking by males to extracts of female gasters and venom glands in an arena assay, but again we did not study copulatory responses. Although venom seems to play a role in mediating courtship or copulation in some paper wasps, it is not known to serve as a sex attractant. In our experiments, we cannot rule out possible female release of alarm chemicals due to handling stress, complicating the interpretation of behavioural assay results, although Freisling (1943) was unable to demonstrate an alarm pheromone in *P. dominulus*.

Polistes dominulus females have ducted type III gland cells in their legs (Beani and Calloni 1991a). *Polistes fuscatus* males responded to female tagmata extracts in a wind tunnel, but the greatest response was to female thorax extract (Reed and Landolt 1990a). During our behaviour bioassays, males responded to extract of ♀ legs in the same manner as to extract of the entire thorax, by rubbing their mandibles and gaster on the substrate. Additionally, males rubbed their gaster on the substrate when exposed to ♀ gaster extract. These behavioural responses have been observed in males of other *Polistes* species and described as scent-marking (Post and Jeanne 1983b; Wenzel 1987). Female odour may not elicit orientation responses, but female extracts of thoraces and legs do appear to elicit scent-marking in males.

Scent-marking behaviour in males is believed to attract females for mating purposes, and to signal territorial ownership to other males (Post and Jeanne 1983b; Beani and Calloni 1991a). Male odour may deter other males of the same species since territorial males will actively chase intruders away and, afterwards, increase grooming and scent-marking behaviour (Post and

Jeanne 1983b; Beani and Calloni 1991a). Males of other *Polistes* species are known to patrol and defend territorial perches near hibernacula and nest sites by gaster dragging and mandible rubbing on the substrate (Post and Jeanne 1983b; Wenzel 1987; Reed and Landolt 1991). The dragging of the male gaster has also been in observed in *Mischocyttarus* spp. (Litte 1979, 1981). Nineteen of 20 males that were presented with ♂ leg extract continuously moved for the two-minute assay; they also rubbed their mandibles on the substrate more often than when presented with the blank. Males rubbed their mandibles on the substrate when presented with ♂ gaster extract. When the seventh sternal gland was dissected and presented to males, they performed gaster dragging in addition to rubbing their mandibles on the substrate. Although males did not respond to ♂ head extract, they did drag their gaster on the substrate when presented with ♂ mandible extract. Therefore, we quantified and report behaviours that are consistent with previous behavioural studies of different *Polistes* species.

The orientation and behavioural evidence reported herein supports the previously stated hypothesis that males use scent-marking for mating and territorial defense purposes by depositing pheromone from well-developed exocrine glands in the gaster, mandibles, and legs onto the perch substrate (Landolt and Akre 1979; Jeanne *et al.* 1983; Beani and Calloni 1991b). The quantification of orientation and behavioural responses to potential sex attractant odours between and within genders is foundational to the larger work of isolating, identifying, and testing sex pheromones in Vespidae.

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